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Safety assessment of food and feed from biotechnology-derived crops employing RNA-mediated gene regulation to achieve desired traits: A scientific review

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ABSTRACT

Gene expression can be modulated in plants to produce desired traits through agricultural biotechnology. Currently, biotechnology-derived crops are compared to their conventional counterparts, with safety assessments conducted on the genetic modification and the intended and unintended differences. This review proposes that this comparative safety assessment paradigm is appropriate for plants modified to express mediators of RNA-mediated gene regulation, including RNA interference (RNAi), a gene suppression mechanism that naturally occurs in plants and animals. The molecular mediators of RNAi, including long double-stranded RNAs (dsRNA), small interfering RNAs (siRNA), and microRNAs (miRNA), occur naturally in foods; therefore, there is an extensive history of safe consumption. Systemic exposure following consumption of plants containing dsRNAs that mediate RNAi is limited in higher organisms by extensive degradation of ingested nucleic acids and by biological barriers to uptake and efficacy of exogenous nucleic acids. A number of mammalian RNAi studies support the concept that a large margin of safety will exist for any small fraction of RNAs that might be absorbed following consumption of foods from biotechnology-derived plants that employ RNA-mediated gene regulation. Food and feed derived from these crops utilizing RNA-based mechanisms is therefore expected to be as safe as food and feed derived through conventional plant breeding.

1. Introduction

RNA interference (RNAi) is an RNA-based mechanism that modulates endogenous gene expression in eukaryotes including plants, insects, fungi, nematodes, and mammals. Because RNAi is a widely occurring biological process, the RNA molecules that mediate this mechanism are a ubiquitous component of the diet for animals including humans. RNAi is mediated by small RNA molecules that bind to and suppress transcription and/or translation of specific messenger RNAs (mRNAs); specificity is driven by base pairing between target mRNAs and these small RNAs. Because of the specificity of RNAi, there is great interest in application of this mechanism for crop improvement and for development of human therapeutics. Based on evidence supporting continual exposure to dietary RNA (including siRNAs, miRNAs, and longer dsRNAs) and biological barriers that limit uptake and biological activity of ingested RNA, there is no reason to expect that consumption of foods or feeds from biotech crops employing traits produced through an RNAi-based mechanism or other RNA-mediated mechanism are any less safe than their conventional counterparts. However, to realize the potential for applications of these mechanisms in agricultural biotechnology, it is necessary to establish scientifically sound principles for evaluating their safety in crop plants. Herein we consider the weight-of-the-evidence supporting the safe use of RNAi in crop plants in the context of the current paradigm for evaluating the safety of biotechnology-derived crops (referred to throughout as biotech crops). This evidence is also considered in the context of one study that suggested oral activity of a plant miRNA after dietary consumption (Zhang et al., 2012a). Based on the weight-of-the-evidence for RNA dietary safety and the robust nature of the current internationally accepted principles for the safety

Abbreviations: CRISPR, clustered regularly interspaced short palindromic repeat; dsRNA, double-stranded RNA; FDA, food and drug administration; LDL, low density lipoprotein; miRNA, microRNA; mRNA, messenger RNA; OECD, organisation for economic cooperation and development; RdRP, RNA-dependent RNA polymerase; RISC, RNA-induced silencing complex; RNAi, RNA interference; rRNA, ribosomal RNA; shRNA, short hairpin RNA; siRNA, small interfering RNA; tRNA, transfer RNA; WHO, World Health Organization.

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evaluation of biotech crops, this review proposes that these principles are applicable to crops modified using RNA-based mechanisms such as RNAi.

2. RNAi: Background and plant applications

2.1. General features of RNAi

Gene suppression was first observed in plants as a cellular mechanism for the recognition and degradation of foreign RNA including viral RNA (Dougherty et al., 1994; Napoli et al., 1990). Fire, Mello, and colleagues defined the RNA-mediated mechanism of gene suppression (i.e., RNAi) in nematodes (Fire et al., 1998), and RNAi-mediated gene suppression has since been observed in fungi, worms, insects, and mammals (Brodersen and Voinnet, 2006; Dykxhoorn et al., 2003; Ghildiyal et al., 2008; Jones-Rhoades et al., 2006; Li and Liu, 2011; Mallory and Vaucheret, 2006; Sandy et al., 2005; Vazquez, 2006). Prokaryotes also utilize RNA-mediated gene silencing through the CRISPR system that is analogous to, but mechanistically distinct from, RNAi (Wiedenheft et al., 2012). Based on these observations, it is apparent that modulation of gene expression through RNA-mediated mechanisms is nearly ubiquitous.

The triggers for RNAi-mediated gene suppression are small double-stranded RNAs (dsRNAs) of 21–27 nucleotides; these small RNAs include small interfering RNAs (siRNAs) and microRNAs (miRNAs) (Hammond, 2005; Zamore et al., 2000). siRNAs and miRNAs are derived from processing of longer dsRNA sequences that do not encode proteins. In plants, biogenesis of siRNAs and miRNAs from precursor dsRNAs involves multiple Dicer-like proteins (Liu et al., 2009), endonucleases that cleave longer dsRNAs. Mature siRNA duplexes contain an interfering antisense or guide strand complementary to a target mRNA sequence and a passenger strand (Caplen et al., 2001; Elbashir et al., 2001; Zamore et al., 2000). RNAi-mediated gene suppression involves incorporation of the guide strand into an RNA-induced silencing complex (RISC) with concomitant degradation of the passenger strand (Tomari and Zamore, 2005). RNAi-mediated gene suppression occurs through either mRNA degradation or translational inhibition (Bartel, 2009; Carthew and Sontheimer, 2009; Fabian et al., 2010; Guo et al., 2010; Huntzinger and Izaurralde, 2011).

2.2. Differences in RNAi mechanisms in different kingdoms

Although the general mechanism of RNAi is conserved across eukaryotes, there are some important phylogenetic differences. A general distinction is that plant miRNAs are usually perfectly or nearly perfectly complementary to their target genes and induce direct mRNA cleavage by RISC, whereas miRNAs in animals trigger either translational repression (Fabian et al., 2010) or target mRNA cleavage (Guo et al., 2010; Huntzinger and Izaurralde, 2011). There is some evidence, however, that miRNAs can also act in the translationally inhibitory mode in plants (Brodersen et al., 2008). Another aspect of RNAi observed in nematodes and plants is intercellular spreading of gene suppression. Classic examples of this phenomenon are systemic transport of viral resistance from a local site of infection to distant sites in plants and systemic spreading observed in *Caenorhabditis elegans* (Jose and Hunter, 2007). The phenomenon of intercellular spreading of RNAi appears to be restricted to plants, fungi, and a subset of invertebrate species (Jose and Hunter, 2007; Voinnet, 2005). Intercellular spreading may be attributed to the activity of RNA-dependent RNA polymerase (RdRP) that is present in plants, worms, and perhaps other invertebrates but does not appear to be present in *Drosophila* or vertebrates (Tomari and Zamore, 2005).

Nematodes also exhibit intercellular and systemic transport of RNA molecules, processes not observed to any significant extent in mammals. RNAi-mediated gene suppression is induced in *C. elegans* by soaking the worms in siRNA-containing solutions (Maeda et al., 2001; Tabara et al., 1998), by feeding bacteria expressing an siRNA to *C. elegans* (Newmark et al., 2003; Timmons et al., 2001), or by injecting RNA isolates from siRNA producing plants into worms (Boutla et al., 2002). There have also been demonstrations of RNAi-mediated gene suppression in larvae of certain species of insects and nematodes upon feeding of plant material engineered to produce dsRNAs targeting genes in these organisms (Baum et al., 2007; Fairbairn et al., 2007; Huang et al., 2006; Mao et al., 2007; Yadav et al., 2006). Although there has been speculation that amplification of the RNAi signal and systemic transport and spreading might be present in mammals under certain environmental conditions (Jose and Hunter, 2007), there is no *in vivo* evidence for these functions in mammals. Taken together with the mammalian barriers to uptake of dietary RNA (Depicted in Fig 1), this information strongly suggests that no adverse effects should be anticipated in mammals following consumption of dietary RNA. This assertion is also discussed below (Section 3.4.5.) in the context of a publication that indicates possible mammalian responses to ingested small RNAs.

2.3. Applications of RNAi in plants

Examples of naturally occurring RNA-mediated gene suppression traits that were selected through conventional breeding include soybean seed coat color (Tuteja et al., 2004) and maize stalk color (Della Vedova et al., 2005), both of which are mediated through suppression of chalcone synthase. Additionally, a conventional low glutelin rice variety resulted from a naturally occurring insertion of a region into the genome that expresses a long dsRNA that suppresses *glutelin* via an RNA-mediated mechanism (Kusaba et al., 2003). RNA-mediated gene suppression has also been leveraged in the production of biotechnology-derived food crops such as

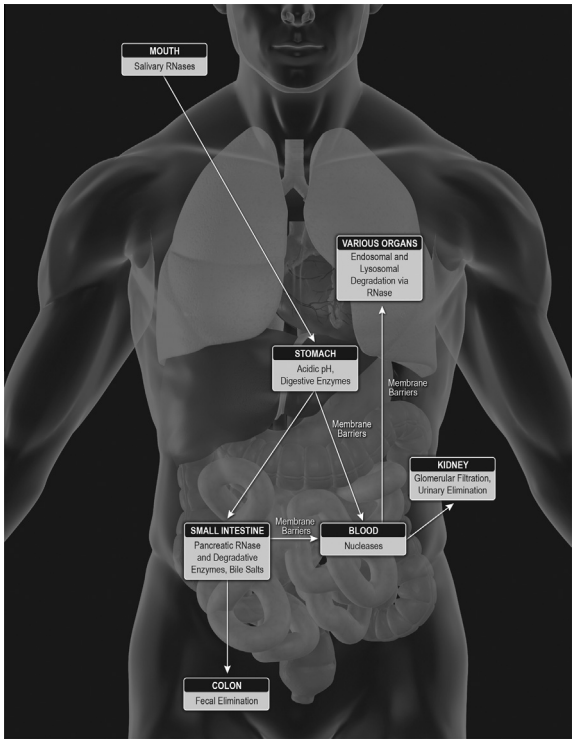

Fig. 1. Biological barriers to uptake and activity of ingested RNA.

virus-resistant squash, papaya, plum, bean, and potato, a delayed ripening tomato, and a soybean with altered oil composition (Frizzi and Huang, 2010; Parrott et al., 2010). RNA-based mechanisms have also been harnessed to improve crop nutritional values (Andersson et al., 2006; Regina et al., 2006), reduce allergen levels (Le et al., 2006), improve agronomic characteristics (Allen et al., 2004; Ogita et al., 2003), and provide insect protection (Baum et al., 2007; Gordon and Waterhouse, 2007; Huvenne and Smagghe, 2010; Mao et al., 2007).

Engineering of plants with traits produced via RNA-mediated gene regulation can be achieved using the same genetic modification techniques used in the production of other biotech crops grown widely today. Plants can be engineered to produce long dsRNAs or short hairpin RNAs (shRNAs) that are processed into siRNAs or miRNAs. Such dsRNA producing constructs are designed to produce transcripts lacking protein-coding open reading frames and translation initiation signals necessary for protein production (Hemmings-Mieszczak and Hohn, 1999; Kozak, 1989). As a result, RNA transcripts produced from the inserted DNA do not give rise to a heterologous protein. Use of RNA-mediated gene regulation (including RNAi-mediated suppression) in biotech crops thus obviates the need to conduct protein safety assessments (Parrott et al., 2010).

3. Safety assessment of food and feed from plants produced using RNA-based traits

3.1. Application of current safety assessment approaches to foods from plants with phenotypes produced through RNA-mediated gene regulation

A well-defined approach already exists for evaluation of the safety of agricultural biotechnology products, namely, the comparative safety assessment paradigm (Codex, 2003; Parrott et al., 2010). This approach is embodied in numerous documents generated by organizations with international oversight, including the World Health Organization (WHO) and the Organisation for Economic Cooperation and Development (OECD), and forms the framework for safety assessment of biotechnology-derived crop products currently sold on the global market. This assessment process addresses safety aspects resulting from intended effects of the genetic modification as well as any unintended effects on the plant that could have resulted from the introduced trait or from the plant transformation process itself. Commercially available biotech crops have been assessed for safety and have received regulatory approvals utilizing this assessment paradigm. The majority of biotech crops currently available in the marketplace have been designed to express one or more proteins that confer some desirable phenotype to the plant (e.g., herbicide tolerance or insect protection). Thus, certain protein-specific aspects of the existing safety assessment paradigm (e.g., homology of the expressed protein to known protein toxins and allergens and digestibility) can rationally be eliminated for traits produced via RNA-mediated gene regulation.

The comparative safety assessment compares the agronomic/phenotypic characteristics and environmental interactions (e.g., crop growth and development, persistence, plant interactions with symbionts, and plant interactions with diseases and insects) and compositional characteristics (e.g., key nutrients, anti-nutrients, and toxins) of the biotechnology-derived crop with those of its conventional counterpart with a history of safe use (Atherton, 2002; Cockburn, 2002; Codex, 2003; EC, 2003; FAO/WHO, 1996; FAO/WHO, 2000; ILSI, 2004; Konig et al., 2004; OECD, 2003). Evaluation of plant morphology and agronomic and phenotypic characteristics is important from the standpoint of marketability and also because this assessment provides a robust, yet very sensitive, screen for any unintended effects of the genetic modification (Cellini et al., 2004; Cockburn, 2002), which in turn can provide a context for results observed in the compositional and nutritional analyses. An understanding of the morphological and agronomic characteristics of a biotech crop compared to its familiar conventional counterpart also provides valuable information for the environmental risk assessment as any unintended differences in these parameters can be assessed for their potential plant pest risk or their potential impact on the biotic or abiotic environment. These aspects of the comparative assessment would also apply to crops with modifications produced through use of RNAi.

Compositional analysis is a key component of the comparative safety assessment as this analysis allows for an evaluation of any unintended changes that may have occurred within the plant as a result of the genetic modification. A series of consensus documents, prepared under the auspices of the OECD, provide a summary of the compositional characteristics of a number of crops with indications of natural variability in key analytes that can be used to facilitate the comparison of a biotech crop and its conventional counterpart and to assess any compositional differences in the context of the range of normal variation (OECD, 2001a,b, 2002, 2005). Evidence to date suggests that there is a high degree of variability in crop compositional data that can be attributed to natural genotypic and environmental variation and that the impact of genetic modification on compositional variability is negligible (Harrigan et al., 2010; Herman et al., 2009; Zhou et al., 2011a).

A scientific evaluation of biotech crop products employing RNA-mediated gene regulation concluded that the comparative safety assessment paradigm is appropriate for evaluating the safety of these crops (Parrott et al., 2010). The safety of biotech crops, including those produced through RNA-mediated gene regulation, has been successfully evaluated using the comparative safety assessment approach as demonstrated by global regulatory approvals of many biotech crops and the absence of confirmed safety incidents as adoption of the technology continues to increase (James, 2011). In addition to standard data utilized in the comparative safety assessment, supplemental data may also include animal feeding studies. In a number of feeding studies with rodents, chickens, livestock, and fish, no difference in nutritional performance has been observed between feeding biotech crops and their conventional counterparts, providing additional supplemental evidence for the safety of crops developed through agricultural biotechnology (Cockburn, 2002; Flachowsky et al., 2005; Hollingworth et al., 2003). For example, a biotech wheat with an RNAi-mediated elevation in amylose content was evaluated in a 13-day rat feeding study with diets containing approximately 50% wheat, and no adverse effects were reported (Regina et al., 2006). Similarly, in a 90-day rat toxicology study, feeding a biotech rice variety with an RNAi-mediated elevation in amylose content elicited no adverse effects at a 70% level of dietary incorporation (Zhou et al., 2011b). Crops such as the Flavr Savr™ tomato and the high amylopectin potato utilize RNA-mediated gene regulation, and animal feeding studies provide supplemental evidence supporting their safety (EFSA, 2006; FDA, 1994; Redenbaugh et al., 1992). Collectively, the weight-of-the-evidence to date supports the safety of biotech crops produced through applications of RNA-mediated gene regulation.

3.2. Differences in assessment of plants that express RNA-producing constructs and those that express engineered proteins

Biotech crops engineered with RNA-mediated gene suppression cassettes are fundamentally different from those containing constructs encoding heterologous proteins because these constructs are intended to express only non-coding RNAs and do not encode protein(s). For plants that express heterologous proteins, the

current safety assessment approach recommends analysis of the introduced protein(s) for any relationship to known protein toxins and allergens. *In vitro* digestibility studies of such proteins are also generally recommended, to evaluate their potential for digestion, as allergenic and toxic proteins may be refractory to digestion (Astwood et al., 1996). Furthermore, acute toxicity of the expressed protein is often assessed because toxic proteins tend to act acutely (Pariza and Johnson, 2001; Sjoblad et al., 1992). Unlike proteins, that can in some rare cases produce oral toxicity, RNA is not known to produce oral toxicity (acutely or otherwise) in humans. According to the US FDA, "Introduced nucleic acids [in biotech crops], in and of themselves, do not raise safety concerns (FDA, 1992)." Furthermore, with regards to RNA-mediated gene regulation in biotech crops, the US FDA goes on to state, "Thus, for example, the introduction of a gene encoding an anti-sense ribonucleic acid (RNA) would not raise concerns about either the gene or the anti-sense RNA. Any safety considerations would focus on the intended effects of the anti-sense RNA." Given the long history of safe consumption of nucleic acids such as RNA, acute oral toxicity studies and evaluation of digestibility of the introduced RNA component are therefore not warranted for plants with introduced RNA-based traits. Any additional studies based on the intended effects of the dsRNA should be considered as needed, on a case-by-case basis.

3.3. Evaluation of unintended effects in biotech crops with RNA-based traits

The evaluation of potential unintended effects in biotech crops (e.g., compositional or agronomic changes) is an important consideration in the comparative safety assessment process (Cellini et al., 2004). Depending on the target gene, biologically meaningful changes in gene expression in a genetically modified plant with a mode of action based on RNAi could be manifested in downstream compositional or agronomic/phenotypic changes to the plant (Parrott et al., 2010). In addition to the intended changes elicited by the expressed RNA, there is a possibility that unintended changes in the plant could result from suppression of genes that were not intended as targets of the expressed dsRNA. These so-called off-target effects would be identified as compositional or agronomic/phenotypic changes that are outside the normal range of variability for these characteristics in the plant species, and once identified must be further assessed for biological significance to food and feed safety. Conventional breeding methods and agronomic/phenotypic selection processes that are also used in the event selection process during the development of biotech crops are stringent tools that help to eliminate undesirable unintended effects resulting from the transformation process (Cellini et al., 2004; Cockburn, 2002; Konig et al., 2004). Furthermore, compositional analysis of food and feed would likely reveal any unintended effects relevant to safety or nutrition of the biotech crop (Hollingworth et al., 2003). There do not appear to be any special considerations that relate to development of RNA-based traits as the general transformation and event selection processes and the comparative safety assessment processes equally apply.

3.4. Evidence supporting safety of oral exposure to RNA

3.4.1. Natural occurrence of long dsRNAs and small RNAs in plants and other foods provides a history of safe use

A history of safe consumption of plant-derived RNAs is evidenced by the fact that all animal and plant-related foodstuffs contain naturally occurring coding RNAs (e.g., mRNAs) and non-coding RNAs. Total plant RNA content is about 1 mg/g of plant tissue (Ivashuta et al., 2009; Lassek and Montag, 1990). Non-coding RNAs including the highly abundant transfer RNAs (tRNAs) and ribosomal RNAs (rRNAs), single-stranded antisense RNAs, as well as the miRNAs and siRNAs that trigger RNAi and their precursor dsRNAs are found in plants (Gould and Francki, 1981; Hamilton and Baulcombe, 1999; Vazquez et al., 2004), including plants used for food such as rice (Fukuhara et al., 1993; Ivashuta et al., 2009; Moriyama et al., 1996; Osato et al., 2003; Wang et al., 2004), *Brassica* species (Cock et al., 1997), *Cucurbit* species (Yoo et al., 2004), and maize and soybean (Ivashuta et al., 2009). Long dsRNAs from exogenous sources are particularly common in plants, including food plants, due to infection from RNA-containing plant viruses (Gould and Francki, 1981). Small RNAs derived from longer non-coding RNAs such as tRNAs and intronic regions may be involved in regulation of gene expression (Rother and Meister, 2011). Animal-derived foods are generally richer in RNA than plant-derived foods, and animal-derived foods are also likely to contribute significantly to overall RNA consumption (Jonas et al., 2001). Animal-derived foods, which also have a history of safe consumption, contain siRNAs and miRNAs that serve a variety of functions, including regulation of endogenous gene expression (Carthew and Sontheimer, 2009).

The fact that small RNAs are safely consumed in human and animal diets is demonstrated by the presence of thousands of small RNAs in rice grain with sequence identity to regions in the human genome and to several livestock animal genomes (Heisel et al., 2008). A subset of rice small RNAs have 100% sequence identity to human transcripts (Ivashuta et al., 2009). Identity between animal small RNAs is higher than the level of identity between plants and mammals and therefore animal small RNAs in the diet are more likely than plant small RNAs to have identity to human transcripts. The safe consumption of small RNAs with a high level of sequence identity to human genes and the ubiquitous role of RNAi in regulation of gene expression in plants and animals demonstrates a history of safe consumption of small RNAs and, by inference, the dsRNA precursors from which they are derived.

3.4.2. Dietary exposure to biotech crop small RNAs

As previously stated, total RNA content in plant-derived foods varies but appears to be on the order of 1 mg/g of tissue (Ivashuta et al., 2009; Lassek and Montag, 1990). Relative percentages (by weight) of the major forms of RNA in plants are roughly 80% rRNA, 3–5% mRNA, and 10–15% tRNA, with small RNAs making up less than 5% of total RNA in plants. Small RNAs in the range of 21–24 nucleotides are present at levels of up to 1.61 μg small RNA/g of conventional soybean grain (average of 0.66 μg/g of grain) and comparable amounts are present in grain from conventional corn and rice (Ivashuta et al., 2009). In tobacco plants engineered to overexpress a dsRNA targeting mouse caspase under the control of a constitutive promoter, siRNA levels in leaf composed about 1.5% of total RNA (Chau and Lee, 2007). Due to the intended overexpression of this dsRNA, this percentage likely represents a higher-end estimate of levels of engineered siRNAs in plants. When taken in the context of the total dietary intake of RNA, the additional RNA from biotech crops will represent a very small fraction of total RNA consumed. Therefore, the application of RNA-mediated gene regulation to biotech crops would not meaningfully impact the levels of dietary purine intake (RNA is a purine source) that can sometimes be associated with aggravation of gout symptoms in gout patients (Zhang et al., 2012b).

Data reported by Ivashuta and colleagues (2009) and by Chau and Lee (2007) were used to make conservative estimates of human dietary exposure to soy and maize small RNAs from hypothetical RNA-based biotech crops (Table 1). Estimates of the highest reported (97.5th percentile) consumption of soybean grain and maize (flour, popcorn, and sweet corn) were obtained from the WHO's GEMS/Food Programme (WHO GEMS/Food, 2013). These values represent a conservative (i.e., high-end) estimate of the maximum amount of a food that would be consumed in a single day in any world area and are intended for use in assessing

Table 1
Sample exposures to plant-derived siRNAs from biotech crops and margins of exposure for humans consuming these agricultural commodities.

Commodity	97.5th percentile commodity consumption (g/kg/day)[1]	siRNA Intake (μg/kg/day)[2]
Soya bean, Dry		
General population	3.03	45
Children ⩽ 6 years	5.55	82
Maize flour		
General population	2.04	30
Children ⩽ 6 years	3.16	47
Popcorn		
General population	3.33	49
Children ⩽ 6 years	3.33	49
Sweet corn (on-the-cob)		
General population	7.16	106
Children ⩽ 6 years	11.52	170

[1] Consumption data from WHO/GEMS Food acute 97.5th percentile estimates of food consumption: http://www.who.int/foodsafety/chem/en/acute_hazard_db1.pdf, accessed January 14, 2013.

[2] Assumes 100% of commodity consumed is from a biotech crop with total RNA levels of 986.6 μg/g grain and that 1.5% of these small RNAs are derived from the transgene (Chau and Lee, 2007; Ivashuta et al., 2009).

potential acute or short-term health risks; estimates are reported for both the general population and for children aged six and under. Using the highest reported levels of grain-derived total RNA per gram of plant tissue observed in the Ivashuta study (986.6 μg RNA/g grain) and a transgene-derived siRNA level of 1.5% of total RNA as reported by Chau and Lee, total exposures to construct-derived small RNAs from a putative biotech soybean product were estimated to be 45 μg/kg/day in the general population and 82 μg/kg/day for children aged six and under. Using the same assumptions cited above for expression levels of plant total RNA/small RNAs for sweet corn (on-the-cob), the corn commodity consumed in the highest amounts, dietary exposure to construct-derived small RNAs from a putative biotech corn product were estimated to be 106 μg/kg body weight/day in the general population and 170 μg/kg body weight/day for children aged six and under. The expression levels of any individual transgene-derived small RNA sequence would be substantially lower than these estimated total amounts. To contrast, doses as high as 10 mg/kg/day of a single chemically modified siRNA are well tolerated in human clinical trials (Vaishnaw et al., 2010) and siRNA doses as high as 200 mg/kg have been systemically injected in rats without adverse effects (Thompson et al., 2012).

Based upon estimated exposures to plant-derived RNA molecules, the minimal oral bioavailability of oligonucleotides (Section 3.4.5.), and the lack of oral toxicity of RNA, margins of exposure for plant-derived siRNAs from consumption of foods and feeds from biotech crops are anticipated to be very large, likely in excess of 10,000 fold. Thus, even upon eating thousands of times more grain than a conservative consumption estimate (i.e., 97.5th percentile) of a putative RNA-based biotech crop – a physical impossibility – no adverse effects due to the exogenous RNA would be anticipated. Although potential risks of a new crop with traits produced utilizing RNA-mediated gene regulation should be assessed, based on the data discussed in this review we propose that minimal risk will result from consumption of small RNAs derived from these biotech crops.

3.4.3. Evidence from mammalian and human studies with siRNA indicates that exposure levels needed to produce systemic effects are greater than those possible from ingestion

The promise of specificity for oligonucleotide therapeutics created great interest in development of drugs that act through an RNAi mechanism (e.g., RNA oligonucleotide drugs); however, systemic delivery of these therapeutics remains a challenge (Behlke, 2006; Nguyen et al., 2008; O'Neill et al., 2011; Vaishnaw et al., 2010). The fact that delivery of functional RNA oligonucleotide therapeutics is one of the major contributors to their limited success to date suggests that biological barriers limit the activity of exogenous RNA in humans and other mammals. In spite of these significant delivery challenges, several RNA drugs are currently undergoing evaluation in Phase II and III clinical trials (Lares et al., 2010; Vaishnaw et al., 2010). About half of these RNA-based drugs are administered locally with the expectation of activity at the site of application (e.g., in the eye, skin, or lung) (Vaishnaw et al., 2010). Oligonucleotide therapeutics delivered systemically have succeeded after extensive efforts to stabilize these oligonucleotides through chemical modifications that impart suitable pharmacokinetic properties and to formulate them in specialized lipophilic delivery vehicles (Behlke, 2006). Doses administered or proposed to be systemically administered to subjects in clinical trials have been as high as 10 mg/kg in the case of unformulated siRNA for prophylactic treatment of delayed graft function in renal transplantation (Quark Pharmaceuticals, 2013) and 1.5 mg/kg in lipid nanoparticles for the treatment of liver cancer patients (Alnylam Pharmaceuticals, 2013). Preliminary readouts indicate good tolerability and movement of compounds into Phase II studies supports the conclusion of safety. The doses used in these therapeutic trials are orders of magnitude greater than anticipated exposures that would result from consumption of food and feed derived from biotech crops employing RNA-mediated gene regulation (see Section 3.4.2.).

The drug delivery challenges experienced to date with development of oligonucleotide-based human therapeutics provide support for the conclusion that small RNA molecules derived from biotech crops employing an RNAi-based mechanism (e.g. siRNAs or miRNAs) are safe to consume. Some key results from mammalian *in vitro* and *in vivo* studies with small RNAs are relevant to safety assessment of biotech crops that utilize a mechanism based on RNAi.

First, siRNAs have relatively high specificity as repeatedly demonstrated by absence of gene suppression by control siRNAs lacking homology to the target gene, as well as a lack of suppression of genes related to the gene being targeted with siRNA (Amarzguioui et al., 2003; Duxbury et al., 2004; Elbashir et al., 2001; Martinez et al., 2002). This implies that in order to induce RNA-mediated gene suppression in the consuming species, assuming that an exogenous dietary RNA could undergo absorption and delivery to potential target cells, the exogenous siRNA would have to have significant sequence identity with a gene in the consuming organism. There is also some evidence to suggest that siRNAs with complementarity to mammalian genes that are produced in and isolated from transgenic plants may not be effective if taken up by mammalian cells, possibly due to a plant-specific siRNA modification (Chau and Lee, 2007).

Second, most mammalian cell types do not efficiently take up double-stranded nucleic acids. A small fraction of siRNAs in circulation or in culture may be transported into cells through endocytosis, but any free nucleic acids that enter cells largely remain within endosomal/lysosomal vesicles and are subsequently degraded by nucleases (Gilmore et al., 2004). Absent transfection reagents, which transiently create pores in cell membranes to facilitate uptake, naked siRNA generally fails to suppress gene expression at concentrations as high as 250 nM (Lingor et al., 2004). RNAi-mediated gene suppression in leukemic B-1 cells was observed without transfection reagents, though this suppression required siRNA concentrations as high as 2 μM (McCarthy et al., 2004). These concentrations are orders of magnitude higher than anticipated exposures resulting from consumption of RNA in

biotech crops, due in part to the limited oral bioavailability of dsRNA molecules (See Section 3.4.5.).

Hydrodynamic injection has been described as an effective method to deliver unformulated siRNA *in vivo* for target gene silencing in mice (Lewis and Wolff, 2007). The procedure requires a high volume solution of siRNA (typically 1–2 ml in mice) and very rapid, high-pressure, intravenous injection of nucleic acid. Functional delivery appears to be restricted to the liver, and delivery efficiency is dramatically reduced if either volume or pressure is lowered. The dose of siRNA and the hydrostatic pressure needed to achieve target gene silencing by unformulated siRNA exceeds what can be encountered through dietary ingestion. In the case of intravenous injection of naked/chemically stabilized siRNAs targeting Apolipoprotein B (no delivery agent and not conjugated to a cholesterol tag), efficacy is not observed in the liver of mice following injection of doses as high as 50 mg/kg (Soutschek et al., 2004). In rats and monkeys, acute toxicity is not observed following i.v. bolus doses of 200 mg/kg and 500 mg/kg of an siRNA targeting p53, respectively (Thompson et al., 2012). A few deaths occurred in rats after single i.v. bolus doses of 1,200 mg/kg (1/10 females) and 2,000 mg/kg (2/10 males). Toxicities observed at 1,200 mg/kg (e.g. erosion and/or ulcers of the glandular stomach and subacute inflammation and edema of the pancreas) were completely or partially reversible following a 2-week recovery period (Thompson et al., 2012). When these study results are considered in the context of the limited oral bioavailability of ingested nucleic acids, the tested doses are quite high (5 orders of magnitude or more) relative to anticipated dietary exposures from biotech crops, making the observed toxicity from intravenous exposures at such doses largely irrelevant to the safety of these ingested nucleic acids. The observed no effect levels also appear to establish a large margin of exposure for orally ingested small RNA molecules from biotech crops. Effective gene suppression or toxicity in an exposed organism following dietary exposure to siRNAs or longer dsRNAs would thus require exceedingly high levels of the RNA and significant sequence identity with the consuming organism, both of which are highly unlikely to result from applications of RNA-mediated gene regulation in biotech crops.

3.4.4. Unmodified siRNAs are metabolically unstable in biological matrices and undergo rapid clearance

In addition to empirical data supporting the history of safe consumption of RNA from plants (and animal products), there is a wealth of data indicating that RNA molecules have a very short half-life in mammalian systems (Behlke, 2006; Molitoris et al., 2009; Thompson et al., 2012; Vaishnaw et al., 2010). RNA molecules have limited stability in biological matrices such as saliva, serum, and plasma. In the case of siRNA duplexes without any chemical modifications, significant degradation occurs in as little as 15 min when the siRNA is incubated in fetal calf or human sera (Haupenthal et al., 2006; Urban-Klein et al., 2005). In the absence of stabilizing modifications, siRNA in human plasma is rapidly degraded with nearly 75% degraded within 2 min (Layzer et al., 2004), most likely due to nucleases and to the chemical environment (e.g. pH). This metabolic instability is one factor that reduces the likelihood that ingested dsRNAs or siRNAs will have biological activity.

Although intravenous injection is the most effective means of delivering a drug to the systemic circulation, because it bypasses the need for systemic absorption, unformulated RNA molecules have been shown to lack systemic activity via this route due in part to rapid renal filtration and excretion (Braasch et al., 2004; Molitoris et al., 2009; Soutschek et al., 2004; Vaishnaw et al., 2010). Although metabolic stability can be altered with chemical modifications (as is the case of the siRNAs in clinical trials), even chemically stabilized siRNAs have limited or no systemic biological activity after injection because of other factors that limit cellular uptake, such as rapid plasma clearance (Vaishnaw et al., 2010).

Because molecules less than the molecular weight of albumin (~67kD) pass through the molecular sieve of the glomerulus, dsRNAs, including siRNAs, are rapidly filtered from blood and excreted in the urine. An siRNA injected into mice exhibited a half-life of 6 min and a high rate of clearance (Vaishnaw et al., 2010). This property of siRNA molecules was confirmed with vital microscopy: siRNA can be visualized in renal filtrate within seconds after injection (Molitoris et al., 2009). Following intravenous injections of an anti-p53 siRNA in rats at doses up to 200 mg/kg, plasma siRNA concentrations declined by >90% within 30 min (relative to levels at 5 min post dose) and declined by >98% within 2 h (Thompson et al., 2012). Only about 1–2% of the intravenously administered dose is absorbed by tissues and the majority of this uptake occurs in the kidney (Thompson et al., 2012). Most of the siRNA is cleared from tissues within 30 h of dosing (below the lower limit of quantification in 6 of 9 extra-renal tissues evaluated) and there is no indication of siRNA accumulation in tissues. These data illustrate the limited biodistribution and the high clearance rate of injected nucleic acids in mammalian systems and demonstrate that most of the material is excreted or metabolized within hours. By inference from the pharmacokinetic properties of injected siRNAs, only a very small fraction of ingested dsRNA or siRNA, should it undergo absorption into the systemic circulation, would be likely to undergo distribution to tissues.

3.4.5. Biological barriers to oral activity of dietary small RNAs and longer dsRNAs

As described above, nucleic acids are ubiquitous components of the diets of nearly all animals. Not surprisingly, there are a number of biological barriers to oral activity of these ingested RNA molecules (Fig. 1). The first barriers encountered by ingested nucleic acids are salivary RNases (Park et al., 2006) and the harsh acidic conditions of the stomach that denature and depurinate nucleic acids (Loretz et al., 2006; O'Neill et al., 2011). Nucleases in the lumen of the gastrointestinal tract and degradative enzymes (and possibly bile salts) from pancreatic secretions into the duodenum also degrade ingested nucleic acids into nutritionally bioavailable nucleotides (O'Neill et al., 2011). The gut also provides a physical barrier to uptake of hydrophilic compounds like siRNAs. Therefore, systemic delivery of RNA via the oral route is difficult to achieve due to rapid degradation and poor transcytosis across the mammalian gut. In the absence of encapsulation to prevent degradation, or addition of chemical stabilization and penetration enhancers, absorption of RNA, including siRNAs, across the gastrointestinal tract is negligible (Akhtar, 2009; Jain, 2008). In addition, the low percentage of ingested nucleic acids that might be systemically absorbed from the gastrointestinal tract must escape nucleases in the blood (Houck, 1958) and renal clearance (Molitoris et al., 2009). In the unlikely event that significant quantities of ingested nucleic acids are absorbed across the gastrointestinal tract and undergo distribution to tissues, in order to affect gene expression these molecules must: (1) cross cellular membranes that pose a significant barrier to uptake; (2) escape from early endosomes to enter the cytoplasm; and (3) avoid degradation by nucleases found within lysosomes (Gilmore et al., 2004; Manjunath and Dykxhoorn, 2010; Sioud, 2005). Each of the above biological barriers would be expected to reduce levels of intact, biologically active siRNAs by an order of magnitude or more such that the cumulative impact of all these barriers would result in an insufficient amount of intact siRNA to impact cellular function. This level of understanding of these biological barriers is consistent with the pharmaceutical industry experiences illustrating limited delivery of therapeutic RNAs.

Although strong evidence provided by pharmaceutical company and academic studies indicates a low potential for oral activity of

ingested RNA molecules, a single study suggested that oral activity may be possible for certain highly expressed plant miRNAs (Zhang et al., 2012a). This study reported that in mice fed a diet consisting entirely of uncooked rice (i.e., human equivalent of about 33 kg/day of cooked rice[1]), several rice miRNAs were detectable in mouse serum and liver. Although the levels of a mouse mRNA with sequence identity to a rice miRNA (miR168a) were not affected following consumption of rice, the authors report that levels of the encoded protein, low density lipoprotein receptor adaptor protein 1, were lower in mice fed raw rice than in mice fed a standard rodent chow diet. This study also reported increases in plasma low density lipoprotein (LDL) cholesterol levels and attributed them to changes in levels of this adaptor protein resulting from rice miR168a ingestion. However, in a 90 day rat feeding study with 70% rice formulated into a nutritionally balanced diet, LDL levels remained unchanged relative to control rats (Zhou et al., 2011b). Additionally, in another study, rats fed 70% rice (formulated into a nutritionally balanced diet) over three generations had reduced LDL levels relative to control rats (Zhou et al., 2012). Blood cholesterol levels are known to be impacted in rodents by differences in dietary composition during fasting and re-feeding (Ryu et al., 2005). The increase in LDL levels observed by Zhang and colleagues most likely resulted from the short-term nutritional impact of consuming only rice following a fasting period, rather than miR168a ingestion, as such a dietary regimen would be lower in fat, cholesterol, and protein than one consisting of a standard rodent chow.

Zhang and colleagues did not report any adverse physiological or toxicological effects, and the observed differences in LDL cholesterol values were not evaluated within the context of historical values for this parameter in mice. Furthermore, this study (Zhang et al., 2012a) was conducted using high levels of rice that were not reflective of anticipated dietary exposure levels and the authors state: "It is unlikely that such high concentrations of mature plant miRNAs can be achieved in serum, plasma, and organs of humans or animals via food intake."

In a study that identified plant miRNAs in publicly available small RNA datasets from mammals (including cultured human cells), chicken and insects (Zhang et al., 2012c), miR168 was highly over-represented even though it is not the most abundant miRNA in staple food/feed crops. Additionally, $\geqslant$96% of miR168 sequences observed in this study were monocot-derived (the sequence is divergent from the dicot sequence), including those from two insects fed only dicot plant materials (Zhang et al., 2012c). The authors of this study therefore concluded that plant miRNAs identified in animal small RNA sequencing data can originate from artifacts of the sequencing process. Although the phenomenon of oral uptake and activity of dietary miRNAs merits further confirmation and evaluation, the weight-of-the-evidence does not suggest that miRNAs derived from normal dietary exposure have a meaningful impact on mammalian or human gene regulation.

Despite extensive pharmaceutical and academic research efforts to develop therapeutics based on modulation of gene expression by siRNAs, there are an extremely limited number of published reports documenting effective oral administration of siRNAs for the modulation of gene expression in mammals and these studies utilize highly specialized carrier molecules (O'Neill et al., 2011). Chemically modified antisense DNA oligonucleotides formulated with caprate, a well-known permeation enhancer, produced average plasma bioavailability of 9.5% in humans across four formulations (Tillman et al., 2008). In the case of a 20-mer DNA oligonucleotide with stabilizing phosphorothioate internucleotide linkages in the backbone, but delivered without a permeation enhancer, oral bioavailability of the radioactively labeled oligonucleotide in rats was only 0.1% (Nicklin et al., 1998). This number is likely to overestimate bioavailability because it was based on measurement of radiolabel rather than intact oligonucleotide (fragments would retain the label) and because the oligonucleotide was chemically modified to enhance stability. In the case of another unformulated phosphorothioate DNA oligonucleotide (i.e., no delivery agent), intestinal absorption of less than 1% of dose was observed in an isolated rat intestinal model (a model that bypasses the harsh conditions of the stomach); most of the labeled oligonucleotide was associated with the outer (epithelial cell) membrane, and very little was localized intracellularly (Khatsenko et al., 2000). Limited oral bioavailability of nucleic acid therapeutics coupled with the presence of internal biological barriers to activity of exogenous RNA (e.g., nucleases, cellular membranes, and endosomes) have made systemic delivery of orally administered oligonucleotide drugs an elusive goal for the pharmaceutical industry. This limited oral bioavailability provides support for our conclusion of oral safety for dietary exposures to small dsRNAs, longer dsRNAs, and other nucleic acids resulting from agricultural applications of RNAi.

In addition to any potential systemic effects of ingested siRNAs and other dsRNAs, the possibility for effects of these dietary constituents on gastrointestinal tissues merits consideration. Available data from single-stranded phosphorothioate DNA oligonucleotide studies demonstrate that oligonucleotides in the intestinal tract are primarily located extracellularly (e.g., in lumen and luminal wall) (Khatsenko et al., 2000; Nicklin et al., 1998) and are thus presumed to be excreted from the body with minimal absorption. Due to RNA degradation by nucleases and biological barriers to cellular uptake, only a very small fraction of ingested nucleic acid (about 0.1% following oral dosing of a DNA oligonucleotide) is absorbed across the intestinal epithelium (Nicklin et al., 1998). Only a very small fraction of the absorbed RNA will escape sequestration by endosomes or degradation by nucleases and become available for RISC incorporation – all prerequisites for RNA-mediated gene suppression. Thus, when metabolism and barriers to RNA absorption are taken into account, it is unlikely that dietary dsRNAs are present in sufficient quantities to mediate systemic effects or local effects on the cells of the mammalian intestinal tract.

3.5. Specificity of RNA-mediated gene suppression

RNA-mediated gene suppression is hybridization-dependent and thus occurs in a sequence-specific manner; however, suppression of genes with less than perfect complementarity has been documented in *in vitro* mammalian screening assays (Jackson et al., 2003; Jackson et al., 2006a; Jackson et al., 2006b; Vaishnaw et al., 2010). Off-target gene suppression can occur through hybridization with genes that have a high degree of sequence similarity to the intended target gene, especially between the siRNA "seed region" (nucleotides 2–8 of the guide strand) and the 3′ untranslated region of an off-target gene (Jackson et al., 2006b). The seed region is critical for mRNA recognition by RISC-incorporated small RNAs. Seed pairing has been shown to be necessary and sufficient for target regulation by some miRNAs (Doench and Sharp, 2004; Krek et al., 2005; Lewis et al., 2005; Lewis et al., 2003), and a single base mismatch within the seed region of the siRNA may eliminate detectable siRNA-mediated silencing of the target (Amarzguioui et al., 2003; Du et al., 2005). However, sequence complementarity outside the seed region is also required for efficient target suppression by siRNAs and some miRNAs (Didiano and Hobert, 2006; Ha et al., 1996; Tay et al., 2008; Vella et al., 2004), and single base mutations outside the seed region can in some cases eliminate

[1] Mice in the Zhang et al. study ate ~7 g of rice per day and this calculation assumes mouse body weight of 30 g. The calculations were made assuming a 55 kg body weight for Chinese adults and 2.57-fold more miR168a in raw rice vs. cooked rice as reported by Zhang and colleagues in their supplementary data; therefore, 7 g rice × 55 kg × 2.57/0.03 kg = 33 kg cooked rice equivalents.

target suppression (Du et al., 2005; Duxbury et al., 2004; Elbashir et al., 2001). In addition, factors such as site context and sequence context contribute to the efficacy of target silencing (Grimson et al., 2007). Therefore, a match between the seed region and a target gene is not necessarily sufficient for gene suppression. The potency of hybridization-dependent off-target gene suppression appears to be several orders of magnitude lower than on-target gene suppression and changes in transcriptional profiles (i.e., off-target regulation of gene expression) have not been shown to impact *in vivo* safety in preclinical studies (Vaishnaw et al., 2010). When these phenomena are considered together with low exposures and biological barriers to ingested RNA, there appears to be a very low likelihood for any toxicologically relevant off-target gene suppression effects in humans or animals due to applications of RNAi or related gene suppression methods in biotech crops.

3.6. Potential for RNA induction of the interferon response

Initiation of the interferon response and inflammatory responses due to siRNA administration have been reported in mammalian *in vitro* systems and following systemic administration of siRNAs in animal models (Judge and MacLachlan, 2008; Robbins et al., 2009). These responses are mediated by receptors that interact with dsRNA such as the Toll-like receptors, the dsRNA binding protein kinase PKR, and the RIG-I and MDA-5 RNA helicases. The induction of this response is influenced by nucleotide sequence and dose of the oligonucleotide, route of delivery, and cell type but interferon induction does not result from gene suppression. Some of these effects appear to be due to liposomal or polycation delivery vehicles and/or chemical modifications to the siRNA backbone, rather than to the siRNA itself (Heidel et al., 2004; Judge and MacLachlan, 2008; Ma et al., 2005). Unmodified dsRNA can trigger an innate immune response in a sequence- and structure-dependent manner (Jackson and Linsley, 2010); however, there are no reports to date of interferon or inflammatory responses following oral exposure to siRNAs or other nucleic acids. In the case of an siRNA delivered orally to mice (encapusulated in a specialized delivery vehicle), immunostimulation was not observed in the presence of target gene suppression (Aouadi et al., 2009). Immunostimulation from an ingested RNA would require absorption of a given RNA to a sufficient concentration for induction of the response, a phenomenon that is improbable given the multitude of biological barriers to attainment of significant levels of systemic RNA after dietary RNA consumption. The history of safe consumption of RNA from a variety of dietary sources also supports the conclusion that immunostimulation following dietary exposure to RNA is highly unlikely.

4. Conclusions

Available data strongly support the conclusion that biotechnology-derived crops employing RNA-mediated gene regulation are safe for human and animal consumption. Nucleic acids are natural components of all foods and feeds and are therefore presumed to be safe based on their long history of safe consumption. There are extensive biological barriers to absorption and cellular uptake of dietary nucleic acids (i.e., components larger than single nucleotides), as well as rapid catabolism and/or excretion of nucleic acids. Preclinical and clinical data on systemic delivery of oligonucleotide therapeutics indicates that cellular uptake is limited and that these agents are generally well tolerated. Therefore, application of the existing comparative safety assessment paradigm to those crops engineered using RNA-mediated gene regulation is appropriate. This safety assessment paradigm involves a comparison of various characteristics of the biotechnology-derived crop with those of the conventional counterpart including characterization of the genetic modification, plant agronomic and morphologic characteristics, and crop composition. A scientifically valid, testable hypothesis should drive any additional safety testing (e.g., animal feeding or additional safety studies) for biotech crops expressing constructs that elicit RNA-mediated gene regulation.

Conflict of interest

All funding for this manuscript was provided by the Monsanto Company. Jay S. Petrick and Brent Brower-Toland are employees of the Monsanto Company. Larry D. Kier and Aimee L. Jackson are private consultants hired by the Monsanto Company. The Monsanto Company is an agricultural company that does research on and markets biotechnology-derived crop products.

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